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(54) Title: ANTI-CD14 ANTIBODIES FOR USE IN THE INDUCTION OF IL-10 SECRETION**(57) Abstract**

The invention relates to anti-CD14 antibodies for use in the induction and/or increase of interleukin-10 secretion and/or induction of immunosuppression, for example for use in the prevention and/or treatment of inflammation. The invention further relates to pharmaceutical compositions for inducing and/or increasing interleukin-10 secretion, comprising anti-CD14 antibodies and/or fragments and/or modified versions thereof, together with a suitable excipient.

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ANTI-CD14 ANTIBODIES FOR USE IN THE INDUCTION OF IL-10 SECRETION

The present invention relates to anti-CD14
5 antibodies for use in the induction and/or increase of
interleukin-10 secretion. The invention further relates to
anti-CD14 antibodies for use in the treatment of
inflammatory conditions based on increase of the IL-10
secretion, and in immunosuppression based on either increase
10 of interleukin-10 secretion and/or the induction of T cell
tolerance and/or anergy.

Interleukin-10 (IL-10) is one of the cytokines and
is produced in the body by a number of different cell types,
mainly by monocytes/macrophages and by some T cells. IL-10
15 may also be produced by non-immune cells, like keratinocytes
and certain tumour cells. IL-10 has an effect on various
target cells. It inhibits the production of pro-inflammatory
cytokines in monocytes and macrophages, exerting an
immunosuppressive and anti-inflammatory effect. Recently it
20 has been found that IL-10 can act directly on T-lymphocytes
(1-5).

The use of IL-10 for treating various medical
indications like septic and toxic shock, tissue rejection,
graft-versus-host disease, acute or chronic inflammation
25 etc. has been described previously. In these applications
IL-10 is always administered systemically (2-23).

This systemic route has various drawbacks. First,
a higher amount than is actually needed should be
administered. Furthermore, it is not yet known which effects
30 IL-10 exerts on locations in the body where it is not
needed. The IL-10 to be administered will almost always
originate from a source which is foreign to the body.

It is a first object of the present invention to
provide a method for effecting a site-specific increase and/
35 or induction of IL-10 secretion leading to anti-inflammatory
effects. Furthermore, the increase and/or induction of IL-10
may contribute directly or indirectly to immunosuppression.

Inflammation is a uniform response of the human body to a variety of stimuli. Phagocytes play a central role in the generation of inflammatory responses by secreting a variety of mediators of inflammation, such as IL-1, IL-6, 5 TNF- α etc.. These mediators act directly on target cells, or indirectly by, for example, attracting other inflammatory cells, and thus contribute to the normal physiological inflammatory response helping the body's immune system to fight invading particles and microbes. However, the 10 mediators may also participate in reactions detrimental to the human body (24-26). Phagocytes can also secrete molecules which exhibit anti-inflammatory effects such as interleukin-10.

The specific immune response acts through various 15 cells. When a foreign particle enters the human body, it will be ingested by specialized cells called phagocytes. Important phagocytes in the human body are monocytes/macrophages (Mo). While circulating in the blood stream these cells are called monocytes, after migrating from the 20 blood stream to the various tissues they are called macrophages. Specialized forms of these cells are the Kupffer cells residing in the liver, lung macrophages, and many more. The foreign molecule (antigen) is digested into small peptides, a process called antigen processing. These 25 small peptides are then embedded in specialized molecules (Major Histocompatibility Complex, MHC) and transported to the cell surface, where the peptides, still embedded in MHC molecules, are presented to other cells (27-29).

T cells have receptors which recognize a complex 30 of a given peptide presented by an MHC molecule. Upon this interaction, the T cells are activated, proliferate and provide help to phagocytes, stimulate the secretion of antibodies, or kill virus-infected or tumor cells. Thus an immune reaction directed specifically against the foreign 35 antigen has been started.

However, the interaction between the T cell receptor on the side of the T cell and an MHC-molecule with its antigen on the side of the antigen-presenting cell (APC)

is not sufficient to activate a T cell. Additional, APC-derived signals are required for initiating T cell activation. The requirement for such a "second signal" has been recognized more than 20 years ago (Bretscher and Cohn).
5 Despite intense research, one could never isolate one single agent acting as specific "second signal", but APC-derived interleukins such as IL-1 and TNF- α have been found to contribute to the activation of T cells (29-33).

Most importantly, if a T cell is activated by
10 engagement of its T cell receptor by MHC+peptide in the absence of APC-derived factors, this T cell not only fails to proliferate, but it remains anergic for subsequent activation, too, even if for this subsequent activation APC contributing appropriate "second signals" are now present
15 (30, 31, 33-40). There are circumstances where induction of lack of immune response may be due to death of the responding T cells, and other circumstances where the T cells may remain alive but are no longer responsive. This in vitro-phenomenon may translate in vivo to tolerance and/or
20 anergy to a given antigen. Induction of tolerance and/or anergy is of great clinical relevance in the context of organ, bone marrow, blood and cell transplantation and in autoimmune diseases.

It is thus another object of the invention to
25 provide a means for inducing immunosuppression on a specific cellular level by the induction of T cell tolerance and/or anergy.

It has now been found that the administration of anti-CD14 antibodies results in various types of effects.
30 Upon triggering with LPS, peripheral blood mononuclear cells (PBMC) secrete TNF- α , IL-8 and other "pro-inflammatory" cytokines. Addition of anti-CD14 antibodies to PBMC triggered by LPS or other stimuli results in inhibition of secretion of TNF- α and in an increased secretion of IL-10.
35 Therefore as a first aspect of the invention by treatment with anti-CD14 antibodies, an induction or increase of IL-10 secretion is achieved. Simultaneously the secretion of TNF- α is downregulated. In some cases anti-CD14 antibodies also

seem to increase IL-10 secretion and reduce TNF- α secretion in cells to which other stimuli such as LPS have not been added.

Furthermore, it was found that the anti-inflammatory effects of anti-CD14 antibodies are not restricted to cellular stimuli known to act through CD14. The pro-inflammatory effects of T cell mitogens, such as superantigens and lectins (for example concanavalin A), which have not yet been described to act through CD14, are also downregulated by anti-CD14 antibodies. Further experiments showed that anti-CD14 antibodies suppress the secretion of cytokines and other mediators of inflammation by monocytes/macrophages and other cells upon triggering with T cell mitogens, such as superantigens and lectins, like concanavalin A. Thus, anti-CD14 antibodies show an effect in the case of triggers that are not mediated by CD14.

Third, it was also found that upon stimulation of peripheral blood mononuclear cells (PBMC) with mitogens simultaneous treatment with anti-CD14 monoclonal antibodies results in inhibition of T cell proliferation and, in parallel, in suppression of the secretion of cytokines derived from antigen-presenting cells, an effect having been described to result in anergy and/or tolerance (30, 31, 33-40).

Direct evidence for an immunosuppressive effect of treatment with an anti-CD14 antibody regardless of the underlying mechanism was provided by an animal experiment. Treatment of rabbits with a monoclonal anti-CD14 antibody results in suppression of both the antigen-specific humoral and the antigen-specific cellular immune response. Observations in vitro concerning the induction and/or increase of interleukin-10 and the downregulation of signals known to enhance the immune response, such as CD80, membrane-bound TNF- α , soluble TNF- α , interleukin-1, interleukin-6, and other factors affecting the immune response, are in agreement with the immunosuppressive effect of anti-CD14 antibody-treatment observed in rabbits.

However, these in vitro studies are not intended to limit anti-CD14-induced immunosuppression to a certain underlying mechanism. Indeed, mechanisms and/or factors influencing the immune response not mentioned herein and/or unknown to date
5 may play a role in anti-CD14-induced immunosuppression. Thus the invention does not rely on a specific mechanism but is based on the effects observed after in vivo and in vitro treatment with anti-CD14 antibodies.

These effects of treatment with anti-CD14
10 antibodies separately or in combination result in anti-inflammatory effects, or immunosuppression, which may both be beneficial for various medical indications.

The invention thus relates to the use of anti-CD14 antibodies in general for immunosuppression by the induction
15 or increase of IL-10 secretion and/or the induction of T-cell tolerance and/or anergy.

CD14 (Mo2, My4, Leu M3) is a myeloid differentiation antigen detected on mature monocytes, macrophages, and on cells from myelomonocytic (M4) and
20 monocytic (M5) leukemias (FAB classification). By contrast, CD14 is not detectable on immature leukemic cells, nor on the human cell lines U937 and HL60. However, both cell lines express CD14 upon stimulation with 1,25-dihydroxyvitamin D₃ or dimethylformamide. Membrane-bound CD14 has a molecular
25 weight of approximately 52 kD. A soluble form of CD14 (molecular weight 48-52 kD) has been detected in supernatants of CD14 expressing cells, as well as in plasma. The CD14 protein has no transmembrane region, but is attached to the cell membrane by a
30 glycosylphosphatidylinositol (GPI) anchor. Interestingly, surface expression of CD14 is deficient in patients with paroxysmal nocturnal hemoglobinuria, an acquired disorder characterized by a selective lack of expression of GPI-anchored proteins. The CD14 gene maps to a region on the
35 long arm of chromosome 5 (5q23-q31) that encodes several myeloid-specific growth factors and growth-factor receptors, including IL-3, IL-4, granulocyte macrophage-colony

stimulating factor, CSF-1, CSF-1 receptor and the receptor for platelet-derived growth factor (41-50).

The IL-10 secretion is site-specific since it will only occur in the proximity of cells having CD14 on their cell surface, or by cells that have been stimulated, for example by LPS.

The various effects of the invention are a general phenomenon observed with various anti-CD14 antibodies, such as 3C10 (ATCC deposit accession number TIB 228), My4 (Coulter), Tük4 (Dako) etc.. However, various anti-CD14 antibodies may have various effects. Some may be more useful for specific applications than others. The skilled person will be capable of determining which anti-CD14 antibody is most suited for a particular purpose.

The induction or increase of IL-10 secretion and/or induction of T cell tolerance and/or anergy may be beneficial for a variety of clinical conditions, such as inflammatory conditions caused by lipopolysaccharide (LPS), including, but not limited to sepsis and septic shock, inflammatory conditions caused by microbial exotoxins such as, e.g., the staphylococcal exotoxins, streptococcal exotoxin, and other toxins, allergic diseases, such as. e.g. asthma, allergic rhinitis, dermatitis, autoimmune diseases, rheumatic diseases, tumors, graft-versus-host-disease following bone-marrow-transplantation, graft rejection following organ transplantation, systemic inflammatory response syndrome (SIRS), pancreatitis, (severe) aplastic anemia, inflammations encountered after burns, trauma, surgery, including prevention and treatment of the capillary leak syndrome, neurological diseases in which immunological mechanisms play a role, including Alzheimer disease, arteriosclerosis, multiple sclerosis, any inflammatory or neoplastic condition not already mentioned above (1-25, 51, 52).

In addition it is known that some malignant cells depend on cytokines, such as IL-1, IL-6 and TNF- α for their growth. IL-10 may inhibit their proliferation. Anti-CD14

antibodies may therefore also be used for treatment of certain forms of malignant diseases.

Furthermore, according to the present invention novel anti-CD14 antibodies are provided. These antibodies
5 were prepared by isolating soluble CD14 by immunoaffinity chromatography using the antibody 63D3 (available from the American Type Culture Collection under accession number HB 44). The antigen was isolated under gentle conditions. In the material isolated CD14-molecules were identified. Using
10 the material isolated by immunoaffinity chromatography containing CD14 molecules monoclonal antibodies were produced. It was found that the antibody was directed towards CD14-bearing monocytes. These novel anti-CD14 antibodies are another candidate for use in the present
15 invention.

The invention further relates to pharmaceutical compositions for treating diseases wherein the induction and/or increase of IL-10 secretion may be beneficial.

Pharmaceutical compositions, comprising one or
20 more anti-CD14 antibodies as the active ingredient for inducing or increasing IL-10 secretion have the form of powders, suspensions, solutions, sprays, emulsions, infusions, aerosols, unguents or creams and can be used for local application, intranasal, rectal, vaginal and also for
25 oral or parenteral (intravenous, intradermal, intramuscular, intrathecal etc.) administration, administration by means of inhalation etc.. Pharmaceutical compositions of the invention can be prepared by combining (i.e. by mixing, dissolving etc.) the active compound(s) with
30 pharmaceutically acceptable excipients with neutral character (such as aqueous or non-aqueous solvents, stabilizers, emulsifiers, detergents, additives), and further if necessary coloring agents and flavoring agents. The concentration of the active ingredient in a
35 pharmaceutical composition can vary between 0.001% and 100%, depending on the nature of the treatment and the method of administration. The dose of the active ingredient that is administered can further be varied between 0.01 μ g and 1 g

per kg body-weight, preferably between 0.1 μ g and 1 mg per kg body-weight.

As the active ingredient the whole antibody may be used, or any fragment of the antibody molecule or molecules
5 derived from the original antibody (e.g. humanized, bispecific or other engineered antibodies and the like) as long as the specificity for CD14 and its IL-10 secretion stimulating or inducing effects and/or its immunosuppressive effects are maintained. The invention is not limited to
10 antibodies, although these are specifically preferred, but also relates to other CD14-binding molecules having the same physiological effect as the antibodies.

The present invention will be further elucidated referring to the following examples, which are only given
15 for illustration purposes and are in no way intended to limit the scope of the invention.

EXAMPLES

EXAMPLE 1

20 Method for the production of novel anti-CD14 antibodies

1. Preparation of CD14 antigen:

Hybridomas 3C10 and 63D3, both secreting anti-CD14 antibodies, were obtained from the American Type Culture Collection (ATCC, MD, USA). The cells were grown in culture
25 and supernatants were collected and frozen. Anti-CD14 antibodies were purified from the supernatants from the hybridomas 3C10 and 63D3 using protein G coupled to Sepharose 4 Fast Flow (Pharmacia) following the manufacturer's instruction. A second purification step was
30 performed using goat-anti-mouse immunoglobulin linked to a Carbolink affinity column (Pierce), following the manufacturer's guidelines. With this affinity column, mouse anti-human CD14 antibodies were purified. During the whole procedure of supernatant production and antibody
35 purification, repeated controls were performed to ensure that the protein isolated was indeed an antibody binding to human monocytes, preferentially to CD14. These controls were performed using flow cytometry, examining by indirect

immunofluorescence whether the supernatant/the purified protein bound to monocytes (since CD14 is expressed only by monocytes).

After resuspension in appropriate buffers, the
5 purified 3C10- and 63D3-antibodies were each coupled to a HiTrap column (CNBr-activated sepharose, Pharmacia), according to the manufacturer's guidelines.

CD14 protein was isolated from human plasma (fresh frozen plasma, Swiss Red Cross). Approximately 15 µg protein
10 per ml of fresh frozen human plasma were obtained. The proteins isolated by affinity chromatography were checked by SDS-PAGE.

2. In vitro immunization:

15 Antibodies directed against monocytes in general and the CD14 protein in particular were produced by in vitro immunization, using the Cell-Prime kit from Immune Systems, Bristol, U.K., according to the manufacturer's guidelines. In this description the terms "anti-CD14 antibodies" and
20 "anti-monocyte antibodies" are used interchangeably since monocytes are the main cell population bearing CD14.

10 µg of CD14 antigen prepared as described above was incubated with a macrophage cell line originating from Balb/c mice, which is part of this kit, in a concentration
25 of 2 µg antigen per ml cell suspension, for 48 hours.

The spleen was removed from a Balb/c mouse after sacrificing the animal, and a suspension of spleen cells was prepared. The spleen cells were added to the macrophage cell line. After addition of another 10 µg of CD14 antigen, the
30 cells were incubated at 37°C for 4 days. Then, the cells were fused with cells of the mouse B-myeloma cell line Ag8 (obtained from Dr. J. Guzman, Infectiology Laboratory, University Children's Hospital, Zurich, and Prof. Hengartner, Institute of Pathology, Zurich University
35 Hospital). After fusion, the cells were distributed in eight 96-well plates for further culture. The cells were kept in HAT (hypoxanthine aminopterin thymidine)-medium, allowing only cells derived from the fusion of a myeloma cell and a B

cell to survive, whereas unfused B cells or myeloma cells died. In 143 of the 480 wells seeded, cell growth was observed. The hybridoma cells from these 143 wells were transferred in 24-well plates cultured in HT-medium for two 5 weeks. Subsequently, IMDM-medium was used.

3. Screening for the production of immunoglobulins of the IgG class

As a first screening step the supernatants of the 10 wells showing cell growth were assessed in an ELISA (Boehringer Mannheim) for the presence of antibodies (regardless of the specificity of these antibodies). Of 122 supernatants tested, all contained immunoglobulins. 7 contained exclusively IgM antibodies, 1 only IgG, and 114 15 both IgG and IgM. 21 hybridomas were directly screened for binding to mononuclear cells.

4. Screening for the binding to normal human peripheral blood mononuclear cells

20 The ELISA described above revealed only the presence of immunoglobulins, but not the specificity of these antibodies. As a second screening step for anti-CD14 antibodies, the supernatants were tested for antibodies binding to normal human peripheral mononuclear cells.

25 Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation from blood of normal volunteer donors. 5×10^5 cells were incubated with the supernatant to be tested. After washing, a second antibody (a rabbit-anti-mouse-IgG antibody) labelled with a 30 fluorescent molecule (FITC) was added in order to detect the first antibody possibly bound to the cells. The fluorescence of the second antibody was then detected by FACS analysis (FACScan, Becton & Dickinson).

22 supernatants were found to contain antibodies 35 binding to human mononuclear cells. Additional 10 supernatants showed borderline results in this assay.

5. Cloning

Cloning the hybridomas was performed by limiting dilution. The cells were diluted to a concentration of 1 cell per ml medium. Subsequently, this cell suspension was
5 cultured in a 96-well plates at 100 μ l per well. Thus statistically, every tenth well contains one hybridoma cell, and the probability of a well containing two cells is only 1:100.

The hybridoma cell lines thus obtained were again
10 subjected to analysis of binding to human PBMC, followed by further rounds of limiting dilution.

Finally, two monoclonal hybridomas were obtained, designated 3B9 and 2D11.

15 **EXAMPLE 2**

The antibodies secreted by hybridoma 3B9 bind to epithelial cells transfected with cDNA for CD14

In this example it was tested whether the antibodies secreted by hybridoma 3B9 specifically recognize
20 CD14 by testing the binding of these antibodies to cells transfected with CD14 by flow cytometry.

1. Methods

The cDNA for CD14 was obtained by polymerase chain
25 reaction amplification and inserted into the plasmid pCEP4 (InVitrogen). 293 cells (embryonal kidney, human: European collection of animal cell cultures (ECACC) No. 85120602) transfected with the α -, β - and invariant chains of human MHC class II were a kind gift of Dr. J. Neefjes, Netherlands
30 Cancer Institute. These cells were additionally transfected with the CD14 cDNA incorporated into the pCEP4 vector by liposomal transfection using Lipofectin (Gibco Life Technologies), Cells successfully transfected were selected by culture in the presence of hygromycin B (100 μ g/ml). Upon
35 flow cytometry analysis using the commercially available anti-CD14 antibodies Leu-M3 (Becton Dickinson & Co.) and IOM-2 (Immunotech) over 70% of the cells transfected with CD14 were found to express the CD14 antigen; by contrast, no

significant staining of 293 wild-type cells nor of 293 cells transfected with the MHC class II α -, β - and invariant chains could be observed. Binding of the antibodies secreted by the hybridoma 3B9 was evaluated by indirect staining using a goat-anti-mouse-FITC antibody (Immunotech) as secondary antibody. Flow cytometry analysis was performed using a FACScan (Becton, Dickinson & Co.).

2. Results

The antibody 3B9 binds to 293 cells transfected with CD14 cDNA, but not to CD14-negative 293 wild-type cells nor to 293 cells transfected with the cDNAs for the MHC class II-molecules as shown in Fig. 6.

15 **EXAMPLE 3**

Effect of anti-CD14 antibodies on cytokine secretion

The effect of treatment with anti-monocyte antibodies on cytokine secretion by cultured human PBMC untreated or stimulated with various doses of LPS was analyzed. Shown here are 5 representative, independent experiments using PBMC from different donors (experiments 1, 2, 3, 4 and 5, depicted in figures 1, 2, 3, 4 and 5, respectively). Furthermore the effect of anti-CD14 antibodies on the secretion of IL-6 upon stimulation with LPS or the toxic shock syndrome toxin-1 (TSST-1) is described.

1. Materials and methods

PBMC were isolated from heparinized venous blood of healthy human donors by Ficoll-Hypaque density gradient centrifugation. The cells were then resuspended in RPMI 1640 supplemented with 10% normal human AB+ serum, or 10% fetal calf serum (FCS), 2 mM glutamine, 50 μ g/ml streptomycin and 100 U/ml penicillin (complete medium). PBMC were cultured at a concentration of 1×10^6 cells/ml. The cells were cultured in 24-well plates for 24 hours. LPS (Sigma) was added in the concentrations indicated in the figures.

Hybridoma supernatants 3C10, 3B9, 2D11 were added at 1:1 (vol:vol). After 24 hours, the cell culture supernatants were harvested and assessed for their content of TNF- α and IL-10 using commercially available ELISAs (R&D Systems) according to the manufacturer's guidelines.

For experiment 5, purified 3C10 monoclonal antibody was used. Purification of the antibody from the hybridoma supernatant was performed using a protein G-column (Pharmacia) according to the manufacturer's guidelines.

10 Purified 3C10 antibody was used at 5 μ g/ml. Hybridoma 3C10 and 63D3 were obtained from ATCC and cultured in tissue culture flasks under the conditions suggested by ATCC.

2. Anti-CD14 antibodies inhibit LPS-induced secretion of
15 TNF- α

Treatment with anti-monocyte antibodies results in downregulation of LPS-triggered TNF- α secretion (Experiment 1, 2a, 3a and 4a). There is a wide variability in the range of the LPS-triggered TNF- α secretion by PBMC from the

20 different donors, a fact well known to researchers working in the field. Furthermore, the base line TNF- α secretion by PBMC not exposed to exogenous LPS varies widely. In experiment 1 (results depicted in figure 1) there is

25 virtually no background secretion, in experiment 2 a small amount, whereas in experiments 3 and 4 secretion of remarkable amounts of TNF- α can be observed when culturing PBMC for 24h without LPS added. This, too, is a result

observed not so rarely when assessing cytokine secretion by PBMC from various donors. The cause of this "background"

30 activation is unclear, it is probable that the cells become activated through the purification procedure. Most importantly, anti-CD14 antibodies also downregulate this type of cellular activation.

35 3. Anti-CD14 antibodies augment and/or induce the
secretion of IL-10

Stimulation of PBMC with LPS is known to result in secretion of IL-10. It was discovered that treatment with

anti-CD14 antibodies results in augmenting IL-10 secretion, as shown in figures 2b, 3b, and 4b. In some experiments (e.g. experiment 4) treatment with anti-CD14 antibodies results in secretion of IL-10 without the PBMC having been stimulated by LPS.

In order to verify that increased secretion of IL-10 upon treatment with anti-CD14 antibodies is not an artefact due to any unrecognized components of the hybridoma supernatant other than the antibody, 3C10 antibodies were purified. Figure 5 shows that purified anti-CD14 antibody, too, increases the amount of IL-10 secreted by PBMC.

4. Anti-CD14 antibodies decrease the ratio $\text{TNF-}\alpha/\text{IL-10}$

It was thus found that treatment with anti-CD14 antibodies has an immunoregulatory effect, in that secretion of the proinflammatory cytokine $\text{TNF-}\alpha$ is downregulated and secretion of the "anti-inflammatory" and "immunosuppressive" cytokine IL-10 is enhanced. To better demonstrate this effect of the anti-CD14 antibodies the ratio of secreted $\text{TNF-}\alpha/\text{IL-10}$ was calculated. It is submitted that this ratio is a useful indicator for the anti-inflammatory effect of these antibodies. The results for the experiments 2, 3 and 4 are depicted in the figures 2c, 3c and 4c, respectively.

5. Anti-CD14 antibodies inhibit secretion of cytokines upon stimulation by triggers other than LPS:

PBMC were cultured for 24 hours at 10^6 cells/ml. Supernatants were harvested after 24 hours and assessed for the presence of $\text{TNF-}\alpha$ by ELISA (R&D). Results, indicated in pg/ml, are listed in Table 1. 63D3 was used as supernatant, 1:1 (vol/vol). SEB (staphylococcal enterotoxin B) (Sigma) was added at 1 $\mu\text{g/ml}$, Concanavalin A (Sigma) at 1 $\mu\text{g/ml}$.

Table 1

	<u>unst.</u>	<u>SEB</u>	<u>Con A</u>
control mAb	77	358	1058
63D3 (anti-CD14)	0	38	173

This experiment shows that treatment with anti-CD14 antibodies not only downregulates LPS-triggered TNF- α secretion; rather, TNF- α secretion is also downregulated when the cells are triggered by SEB, serving as one example for a superantigen, or by Concanavalin A, one example for a non-microbial stimulus.

6. Treatment with anti-CD14-antibody inhibits toxin-triggered increase of secretion of interleukin-6 by normal human PBMC

PBMC were isolated from heparinized venous blood from a healthy human volunteer donor, and cultured for 24 h with the stimuli and antibodies indicated. My4 (Coulter) and control IgG2 (R&D) were used at 25 μ g/ml, T α k4 (Dako) at 3.6 μ g/ml. Interleukin-6 was determined by ELISA using a kit from Boehringer-Mannheim following the manufacturer's guidelines. Table 2 shows the results.

Table 2

20

Interleukin-6 (pg/ml)		
Treatment:	<u>no</u>	<u>My4</u>

LPS, 1 ng/ml	441	208
25 TSST-1, 100 ng/ml	446	140

Background production of interleukin-6 in unstimulated cells was 17 pg/ml.

30 This example shows that treatment with an anti-CD14 antibody inhibits cellular activation not only when triggered by LPS, but also when triggered by other stimuli, such as the superantigen TSST-1. Treatment with an anti-CD14 antibody furthermore results in prevention of upregulation and/or downregulation of secretion of interleukin-6, a molecule involved in stimulation of immunoglobulin-secretion by B cells.

Interleukin-6 is an important trigger for secretion of immunoglobulins by B cells; prevention of upregulation and/or downregulation of secretion of interleukin-6 thus further supports the observation that treatment with anti-CD14 antibodies results in suppressing the immune response, not only by inhibiting T cell proliferation, but also by indirectly affecting secretion of immunoglobulins.

10 **EXAMPLE 4**

Other effects of treatment of cells with anti-CD14-antibody

Treatment with anti-CD14-antibodies not only results in a modified cytokine secretion but also in various other effects, such as an upregulation or downregulation of the expression of the surface molecules.

1. Materials and methods

1.1. Isolation of blood cells

Unless specified otherwise the following materials and methods were used in the experiments listed below.

Peripheral blood mononuclear cells (PBMC) and monocytes were prepared as described elsewhere (53). Briefly, peripheral blood mononuclear cells were isolated from venous blood of healthy human donors by Ficoll-Hypaque density gradient centrifugation. The cells were then resuspended in RPMI 1640 supplemented with 10% AB+ serum. For isolation of monocytes, the cells were adhered for 1 h at 37°C in a 5% CO₂ atmosphere in 100 x 15 mm plastic Petri dishes (Falcon), each containing 10-15x10⁷ mononuclear cells. After removing the non-adherent cells, the dishes were extensively washed with warm medium, then incubated with cold PBS on ice for 15 min. Adherent monocytes were subsequently recovered by vigorous pipetting, washed and resuspended in complete medium at 1x10⁶ cells/ml. Cell viability, as determined by trypan blue exclusion, was always > 95%.

1.2. Cell culture

Cells (PBMC or purified monocytes, as indicated) (1×10^6 cells/ml) were incubated in medium alone, or in the presence of stimuli as indicated. Toxic shock syndrome toxin-1 (TSST-1) was used at 100 ng/ml, staphylococcal toxin B (SEB, Sigma) at 1 μ g/ml unless otherwise indicated. Where indicated antibodies have been added: control antibodies (IgG2, R&D), or My4 (anti-CD14, Coulter; dialyzed to remove azide) or 63D3 (anti-CD14, ATCC, purified using a protein A column), or Tük4 (anti-CD14, Dako). Unless otherwise indicated antibodies were used at 25 μ g/ml final concentration, except for the Tük4, which was used at 3.6 μ g/ml.

1.3. Flow cytometry analysis

Cells in staining buffer (RPMI 1640- 2.5% FCS, containing 0.01% sodium azide) were incubated with appropriate dilutions of FITC- or phycoerythrin-conjugated antibodies for 40 min at 4°C. Antibodies used were: anti-membrane TNF- α mAb (R&D); anti-CD80 (Ancell). PE- and FITC-conjugated murine IgG mAbs of unrelated specificity (Becton Dickinson & Co.) were used as controls. The cells were then extensively washed and fixed in paraformaldehyde. For indirect immunofluorescence, cells were first incubated with the primary antibody as indicated above, then washed and subsequently incubated with a FITC-labeled goat-anti-mouse antibody (Immunotech). Percentages of positive cells and mean fluorescence intensity (MFI) were analyzed by a FACScan (Becton Dickinson & Co.); for analysis of expression of molecules on monocytes a gate on the monocyte population was applied, as defined by forward and side light scatter. Five thousand cells were counted.

1.4. Determination of cytokin levels

Levels of cytokin s were determined using commercial ELISA kits (R&D) following the manufacturer's guidelines.

2. Treatment with anti-CD14-antibody inhibits toxin-triggered upregulation of CD80 in normal human monocytes

CD80 (also denominated B7) is a molecule expressed on antigen presenting cells. By interacting with the CD28-antigen expressed on T cells it delivers a costimulatory signal critical for activation of T cells. Inhibiting this interaction by supplying a soluble ligand for CD80 (such as CTLA4Ig) competing with CD28 for binding to CD80 results in inhibition of delivery of a second signal necessary for T cell activation and hence anergy and/or tolerance and/or immunosuppression (54).

In this example it was assessed whether treatment with anti-CD14 antibodies affected expression of CD80 by normal human monocytes.

Normal human PBMC were isolated from venous blood by Ficoll-Hypaque density gradient centrifugation. PBMC were cultured at 1×10^6 cells/ml medium (RPMI 1640, supplemented with 10% human AB+ serum) alone, or in the presence of stimuli (TSST-1; Sigma; 100 ng/ml; SEA; 1 μ g/ml). Where indicated anti-CD14 antibodies (My4, Coulter; 25 μ g/ml) have been added. After 24 h the cells were harvested, and stained for CD14 (anti-CD14-FITC antibody from Becton, Dickinson & Co.; isotype control). After staining expression of these antigens were assessed by flow cytometry gating on the monocyte population as defined by forward and side scatter. Treatment of the cell cultures with control antibodies (IgG2, R&D; 25 μ g/ml) did not influence toxin-triggered upregulation of CD80-expression. The results are shown in table 3.

Table 3

CD80-expression on monocytes (MFI)

	<u>no ab</u>	<u>My4</u>
unstimulated	160	226
TSST-1	360	240
SEA	364	225

Indicated is the mean fluorescence intensity (MFI) measured as described above. MFI is a marker for the number of molecules expressed on the cells, i.e. the higher the MFI, the more molecules are expressed on the cells.

- 5 Stimulation of PBMC by the toxins listed resulted in upregulation of the CD80 antigen. Treatment with My4, an anti-CD14-antibody, prevented upregulation of CD80.

This example shows that treatment with anti-CD14-antibodies inhibits cellular activation (in the form of CD80
10 expression) not only when triggered by LPS, but also when triggered by other stimuli, such as the superantigens TSST-1 or SEA, and results in a prevention of upregulation and/or a downregulation of CD80. Since CD80 is a signal required for activation of T-cells a decrease in its expression may
15 result in an immunosuppressive effect.

3. Treatment with anti-CD14 antibodies prevents
 superantigen-triggered upregulation of membrane-TNF- α

Stimulation of normal human monocytes with the
20 superantigens toxic shock syndrome toxin-1 (TSST-1) and staphylococcal exotoxin B (SEB) results in upregulation of membrane-bound TNF- α , as indicated by an increase in fluorescence intensity upon flow cytometry analysis as shown in Fig. 7. Treatment with anti-CD14 antibodies inhibits
25 TSST-1- and SEB-triggered upregulation of membrane-TNF- α .

This example again shows that treatment with anti-CD14-antibodies inhibits cellular activation not only when triggered by LPS, but also when triggered by other stimuli, such as the superantigens TSST-1 and SEB. Furthermore it
30 follows that treatment with anti-CD14-antibodies results in prevention of upregulation and/or a downregulation of membrane-TNF- α , a signal involved in activation of T-cells.

Activated T cells express receptors for TNF- α , and TNF- α enhances the T cell response (56, 57); thus, by
35 influencing TNF- α -production, treatment with anti-CD14 antibodies will also affect the antigen-specific immune response.

4. Effect of treating PBMC from a patient with septic shock in vitro with anti-CD14 antibodies

Venous blood was taken from a patient with septic shock. Blood cultures were positive for meningococci, and the patient suffered from multiorgan failure. 5 days after admission to the hospital, venous blood was taken and peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. PBMC were cultured at 1×10^6 cells per ml medium (RPMI 1640, supplemented with 10% AB+ human serum). No exogenous stimuli were added to the cell cultures. The cells were cultured in medium alone, or with the addition of antibodies (control IgG1 and IgG2, 25 μ g/ml; R&D), IOM2 (anti-CD14, 25 μ g/ml; Immunotech); My4 (anti-CD14, 25 μ g/ml; Coulter), 2D11 (anti-CD14, produced in our laboratory, supernatant, 1:1 vol/vol). After 24h of culture, the supernatants were harvested, and levels of TNF- α were determined by ELISA (R&D). The results are shown in Figure 8.

PBMC purified from this blood sample secreted upon in vitro culture significant levels of TNF- α , despite the fact that at the time of blood sampling the patient had received antibiotic treatment for 5 days and LPS was not anymore detectable in the patient's serum at this time point. In vitro-treatment with anti-CD14 antibodies resulted in downregulation of this TNF- α -secretion. In the absence of detectable LPS-levels in the patient's serum this effect of anti-CD14 antibodies cannot easily be explained by blocking CD14 (CD14 being the receptor for LPS); rather, treatment with anti-CD14 antibodies results in deactivation of monocytes/macrophages.

The examples showing a prevention of upregulation and/or a downregulation of membrane-TNF- α and of CD80 by treatment with anti-CD14 antibodies further support our observation that treatment with anti-CD14 antibodies suppresses so called "second signals" provided by antigen-presenting cells (APC) which are crucial for activation of T cells. As discussed above, if an APC only presents a peptide in the context of MHC molecules ("first signal") to a T cell

but fails to provide costimulatory signals ("second signals") the T cell will not become activated but rather "anergic".

Thus, treatment with anti-CD14 antibodies
 5 downregulates and/or prevents upregulation of various second signals (e.g. membrane-bound molecules such as CD80, membrane-bound molecules such as TNF- α , IL-1, etc.) normally provided by resting and/or activated antigen-presenting cells. This results in immunosuppression and/or anergy
 10 and/or tolerance, as discussed above.

5. Treatment with anti-CD14 antibodies of normal human PBMC preactivated with LPS results in deactivation of the cells, even if at the time of antibody-treatment no
 15 exogenous stimulus persists

THP-1 cells were obtained from ATCC. THP-1 cells were cultured for 5 days in the presence of vitamin D3 (Calciex, Roche: 10^{-8} M). The cells were stimulated with LPS (1 ng/ml; Sigma) for 6h. After extensive washing the cells
 20 were resuspended in medium (RPMI 1640, supplemented by 10% FCS) with the addition of My4 (anti-CD14 antibody, Coulter; 25 μ g/ml), or with the addition of 3C10 (anti-CD14 antibody, ATCC; supernatant, 1:1 vol/vol), or with the addition of control antibody (mix of IgG1 and IgG2, each 20 μ g/ml; R&D),
 25 and cultured for another 20 h. Then the supernatants were harvested and the content of TNF- α was determined by ELISA (R&D). No LPS was added for the second incubation. Were indicated, polymyxin B was added to neutralize LPS which might have persisted despite the washing procedure. Table 4
 30 shows the results.

Table 4

	TNF- α (pg/ml)		
	treatment:	<u>control ab</u>	<u>My4</u> <u>3C10</u>
35 -----			
no polymyxin		58	<10 <10
polymyxin		65	<10 <10

Treatment of pretreated THP-1 cells with anti-CD14 antibodies results in downregulation of TNF- α secretion, even if potentially contaminating LPS was neutralized by polymyxin. This example shows that the effect of treatment
5 with anti-CD14 antibodies is not limited to prevent LPS-triggered activation by blocking the LPS-receptor CD14, but rather results in deactivation of THP-1 cells.

6. Anti-CD14 antibodies inhibit the physical interaction
10 between CD14 and MHC class II-molecules, and lack of
MHC class II-molecules results in vivo in an increased
IL-10-production in response to LPS

There is general agreement that the LPS-receptor CD14 has to interact with other molecules proposed to serve
15 as signal transducers following engagement of CD14 by LPS and/or by LPS-binding protein. We have previously shown that

i) treatment of PBMC with anti-CD14 antibodies results in an increased secretion of IL-10 in response to LPS;

20 ii) MHC class II-molecules participate in signal transduction upon engagement of CD14 by LPS and/or LPS-binding protein;

iii) - at least some - anti-CD14 antibodies interrupt the physical interaction between CD14 and MHC
25 class II-molecules. These observations are described in co-pending application PCT/EP95/05164.

Thus interrupting the signal transduction pathway activated by stimulation with LPS between CD14 and MHC class II-molecules by "removal" of MHC class II-molecules should
30 result in increased secretion of IL-10 in response to LPS, too.

To test this hypothesis we challenged MHC class II-positive C57BL/6 mice and MHC class II-knock out mice (B6AAO) in vivo with LPS and compared the serum levels of
35 IL-10 2h after LPS-injection.

C57BL/6 mice and B6AAO mice (MHC class II knock-out mice; a kind gift of the Basel Institute for Immunology; ref. 55) were injected with 2.8 mg/kg of LPS (E.coli

0111:B4) diluted in sterile NaCl 0.9%, or with 0.9% NaCl only. 5 mice for each group were examined. After 2 h, the mice were sacrificed and bled. The blood was allowed to coagulate at room temperature and subsequently centrifuged to obtain the serum. Levels of IL-10 were determined in the serum by ELISA (Biosource), following the manufacturer's guidelines. The mean +/- standard error of the levels of IL-10 measured in the sera from the 5 mice of each group is indicated in table 5.

10

Table 5

		IL-10 (pg/ml):	
		<u>NaCl</u>	<u>LPS</u>

15	C57BL/6 mice:	12 +/- 3	150 +/- 9
	B6AAO mice:	17 +/- 3	416 +/- 37

Presented are the mean +/- standard error of the levels of IL-10 in the sera from 5 mice for each group.

20 This example shows that MHC class II-negative mice produce markedly more IL-10 in response to LPS in vivo, thus confirming our hypothesis in vivo.

EXAMPLE 5

25 Addition of neutralizing anti-interleukin-10 antibody (partially) reverts anti-CD14-induced inhibition of T cell proliferation

Peripheral blood mononuclear cells were isolated from blood of healthy volunteer donors as described for the other examples. PBMC were cultured in RPMI 1640 supplemented with 10% normal human AB+ serum in a 96 well plate (0.2x10⁶ cells/well). Cells were stimulated with staphylococcal exotoxin B (SEB, 1 µg/ml; Sigma). The anti-CD14 antibodies (My4, Coulter; Tük4, Dako) and the control antibody (IgG2, R&D) were added at 25µg/ml. Neutralizing anti-interleukin-10 antibody (R&D) was used at 100µg/ml. After 3 days of culture, ³H-thymidine was added before harvesting the cells after another 8h. ³H-thymidine incorporation was measured by

scintillation counting. Data are presented in Fig. 9 as percentage of the dpm observed in the sample stimulated with SEB without any antibody added (100%). Cell proliferation in the wells without SEB was <7% with or without antibodies added.

Addition of anti-IL-10 antibodies results in general in upregulation of proliferation.

EXAMPLE 6

10 Anti-CD14 antibodies affect immune response in vivo

This example was performed to study immunomodulating effects of a monoclonal antibody against human CD14. As this monoclonal antibody cross-reacts with rabbit CD14 this example was performed in rabbits.

15 The aim of the example was to investigate whether intravenous anti-CD14 administration affects the immune response induced after an (primary or secondary) ovalbumin immunization. Effects of anti-CD14 administration on in vitro ovalbumin or mitogen induced lymphocyte proliferation, 20 in vivo produced (ovalbumin specific) IgG levels and in vivo Delayed Type Hypersensitivity response against ovalbumin were measured.

1. Materials and methods

25 1.1. Test substances

The test monoclonal antibody (test Mab) was a monoclonal Mouse Anti-Human Monocyte CD-14, Clone TÜK4 (Dako), Ig fraction, without sodium azide.

The control monoclonal antibody (control Mab) was 30 monoclonal Mouse IgG2a (R&D), anti-Keyhole Limpet Hemocyanin (KLH), code No. MAB003,

As the ovalbumin for i.v. immunization Inject® Ovalbumin, product number 77120, Pierce, Rockford III., U.S.A. was used.

35

1.2. Animals

The study was performed with 20 Specific Pathogen Free (SPF)-bred New Zealand White albino rabbits obtained

from Broekman Instituut B.V. Nederland, Someren, the Netherlands. Upon arrival, the animals were taken in their unopened shipping containers directly into the animal room assigned to the study. The animals were checked for overt
5 signs of ill health and anomalies and were placed, individually, in suspended galvanized cages, fitted with a wire-mesh floor and front. Blood was collected from 2 arbitrarily chosen animals and used for serological control. The results of the serology were reported to be negative and
10 specific measures related to the quarantine procedures were withdrawn.

Housing conditions were conventional. Feed and water were provided ad libitum. The general condition and behaviour of all animals was checked and recorded daily.
15 Body weight were recorded at days 0, 7, 15, 21 and 28 of the study.

At the start date of the study, (day 0) the rabbits were allocated to four groups (A-D), proportionately by weight class and each animal was uniquely identified by
20 an animal identification number applied with a marker pen in the ear. The different groups were treated according to the design presented in table 6 and as described hereunder.

Blood samples were collected into two sterile 10 ml lithium heparin tubes (vacutainer, Becton Dickinson,
25 Meylan, France) from the ear artery at days 0, 15 and 28 of the study.

All animals received an intravenous injection with Mab (dose: 0.2 mg/kg body weight) just before one of the ovalbumin immunizations. At day 0, the first control group
30 (A) received an i.v. injection with the control Mab (see 1.1) directly following the primary ovalbumin immunization. The two other groups (B and D) did not receive a Mab injection following the primary ovalbumin immunization. At day 15, the second control group (B) received an i.v.
35 injection with the control Mab (see 1.1) directly following the secondary ovalbumin immunization. The second treatment group (D) received an i.v. injection with the test Mab (see 1.1) directly following the secondary ovalbumin

immunization. The other groups (A and C) did not receive a Mab injection at the secondary ovalbumin immunization.

Table 6

5 Design main study

(all actions are placed in chronological order)

All groups:	Control group A	Control group B	Treatment group C	Treatment group D
	Administered Mab iv:			
10 Day 0: ↓ Blood sampling ↓ ↓ DTH id* ↓ OVA sc** ↓	Control	No	Test	No
15 Day 15: ↓ Blood sampling ↓ ↓ OVA sc** ↓	No	Control	No	Test
20 Day 28: ↓ Blood sampling ↓ DTH id*				

* DTH id = Delayed Type Hypersensitivity assessment

** OVA sc = Subcutaneous Ovalbumin injection

25 Ovalbumin immunizations were performed with doses of 300 µg in 500 µl 0.15 M NaCl solution at days 0 and 15. Each immunization consisted of two subcutaneous injections, at both upper axilla, with a volume of 250 µl each. At day 0 and day 28 (all animals were intradermally injected with 50
30 µl of 0.15 M NaCl containing different doses of ovalbumin (vehicle control: 0 µg or 3 µg or 10 µg or 30 µg ovalbumin) on a shaven flank to assess the DTH response after 24 hours. All actions were performed in the following order: blood sampling for all animals (before 9.00 a.m.) followed by, if

applicable, injection of the appropriate Mab, DTH injection i.d. and ovalbumin immunization s.c. (between 9.00 and 11.00 a.m.).

PBMC were used for the proliferation assays, 5 plasma samples were stored at -20°C until used for the determination of ovalbumin specific IgG.

1.3. Immunoglobulin assays

Ovalbumin-specific IgG antibodies were measured in 10 the plasma samples obtained at days 0, 15 and 28 using a sandwich ELISA. Briefly, flatbottom microtiter plates (NUNC Immuno Plate, Roskilde, Denmark) were coated overnight (4°C) with 100 µl/well ovalbumin diluted to 5 µg/ml in carbonate buffer pH 9.6. Next, per well 100 µl of two dilutions of the 15 plasma (1/30,000 and 1/60,000) or 11 dilutions of a reference pooled rabbit plasma (dilution range: 1/16,000 - 1/512,000) and a reagent blank (Phosphate Buffered Salt (PBS)/1% Bovine Serum Albumin (BSA)) were added in duplicate and the plates were incubated for 45 minutes at 37°C. After 20 washing with PBS/1% BSA per well 100 µl of peroxidase conjugated anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) 1/8,000 diluted in PBS/1% BSA. After 30 minutes incubation at 37°C and subsequent washing with PBS/ 1% BSA 100 µl 3,3',5,5' Tetra Methyl Benzidine 25 substrate we added. After an incubation period of 20 minutes at 37°C the colour reaction is stopped by addition of 100 µl of 2N H₂SO₄. Optical densities are measured at 450 nm using a Bio Rad microplate reader 3550 (Bio Rad Laboratories, Richmond, CA). Based upon the standard curve obtained with 30 the reference pooled rabbit plasma, containing an assumed number of 1,000 U/ml IgG, concentrations of IgG in the plasma samples were determined and expressed in U/ml.

2. Results

35 2.1. Clinical signs and body weights

No changes in general condition or behaviour due to the Mab injections, ovalbumin immunizations or otherwise were noted.

At day 0 of the study the mean body weight was 1883 gram (based on all animals). The average weight gain per animal was 203 gram/week. No differences in body weight gain between the test groups and their matching control 5 groups were observed.

2.2. Ovalbumin specific IgG

Table 7 shows group means (n=5) of the Ovalbumin specific IgG ELISA performed using plasma samples obtained 10 at days 0, 15, 28.

Table 7

Group	Ovalbumin specific IgG (U/ml) Day 0	Ovalbumin specific IgG (U/ml) Day 15	Ovalbumin specific (U/ml) Day 28
15 A	4	16	48040
B	4	34	47360
C	4	25	28400
D	9	27	25020

20 2.3. Delayed type hypersensitivity (DTH)

Table 8 shows DTH responses measured 24 hours after intradermal ovalbumin challenges at day 28 of the study. Presented are the numbers of animals which showed an induration and/or oedema at the injection site.

Table 8

Group	Day 0	Day 29
A	0/5	4/5
B	0/5	3/4*
C	0/5	1/5
D	0/5	0/5

* 1 animal not determined

3. Conclusion

The immunosuppressive effect of treatment with anti-CD14 antibodies was thus demonstrated in an animal model in vivo. The results show that treatment with anti-CD14-antibodies suppresses both the antigen-specific humoral immune response (ovalbumin-specific IgG production in our example) as well as the antigen-specific cellular immune response (delayed type hypersensitivity) in vivo.

REFERENCES

1. Fiorentino, D.F., et al., (1989), J. Exp. Med. 170:2081-2095
2. Goldman, M., et al., (1994), Rev. Med. Brux. 15:61-65
3. Moore, K.W., et al., (1993), Ann. Rev. Immunol 11:165-190
4. Mosman, T.R., (1994), Immunol. 11:165-190
5. St. Georgiev, V., and Albright, J.F., (1993), Ann. NY Acad. Sci. 685:584-602
6. Bogdan, C., and C. Nathan, (1993), Ann. NY Acad. Sci. 685:713-739
7. Caux, C., et al., (1994), Int. Immunol. 6:1177-1185
8. Delvaux, A., et al., (1994), Transplantation 58:972-974
9. Enk, A.H., et al., (1994), Exp. Med. 179:1397-1402.
10. Florquin, S., et al., (1994), I. Immunol. 153:2613-2623.
11. Fluckiger, A.C., (1994), J. Exp. Med. 179:91-99.
12. Gorczynski, R.M., and Wojcik, D., (1994), J. Immunol. 152:2011-2019
13. Holland, G., and Zlotnik, A., (1993), Cancer Invest. 11:751-758
14. Li, L., et al., (1994), Immunol. 153:3967-3978
15. Marchant, A., et al., (1994), Prog. Clin. Biol. Res. 388:295-306
16. Montaner, L.J., et al., (1994), J. Gen. Virol. 75:3393-3400
17. Montz, F.J., et al., (1994), Fertil. Steril. 61:1136-1140
18. Penniine, K.J., et al., (1994), Clin. Immunol. Immunopathol. 71:169-175
19. Rott, O., et al., (1994), Eur. J. Immunol. 24:1434-1440
20. Wang, L., et al., (1994), Cell Immunol. 159:152-169
21. Wang, P., et al., (1994), Blood 83:2678-2683
22. Tumpey, T.M., et al., (1994), J. Immunol. 153:2258-2265
23. Willenborg, D.O., et al., (1995), Scand. J. Immunol. 41:31-41
24. Vasalli, P., (1992), Ann. Rev. Immunol. 10:411-452

25. Strieter, R., et al., (1993), Crit. Care Med. 21:447-463
26. Bone, R. (1991), Chest. 101:802-808
27. Germain, R.N. and Hendrix, L.R., (1991), Nature 353:134
28. Sadegh-Nasseri, S., and Germain, R.N., (1992), Immunology Today 13:43
29. Unanue, E.R., and Cerottini, J.C., (1989), FASEB Journal 3:2496
30. Bretscher, P.A., and Cohn, M., (1970), Science 171:1042-1049
31. Bretscher, P., (1992), Immunology Today 13:74-76
32. Miller, J.F.A.P., and Morahan, G., (1992), Ann. Rev. Immunol. 10:51-69
33. Weaver, C.T., and Unanue, E.R., (1990), Immunolgy Today
34. Jenkins, M.K. & R.H. Schwartz (1987) J. Exp. Med. 165:302-319
35. Lafferty, K.J. et al. (1992) Progr. Immunol. 6:1040-1054
36. Mueller, D.L. et al. (1989) Ann. Rev. Immunol. 7:445-480
37. Hewitt, C.R.A. et al. (1992) J. Exp. Med. 175:1493-1499
38. Quill, H. and R.H. Schwartz (1987) J. Immunol. 138:3704-3712
39. Solbach, W. et al. (1991) Immunology Today 12:4
40. Weaver, C.T. et al. (1988) Proc. Natl Acad. Sci USA 85:8181-8185
41. Bazil, V. et al. (1986) Eur. J. Immunol. 16:1583-1589
42. Ferrero, E. & S.M. Goyert (1988) Nucleic Acids Res. 16:4173
43. Ferrero, E. et al. (1990) J. Immunol. 145:331-336
44. Goyert, S. et al. (1988) Science 239:497
45. Griffin, J.D. et al. (1981) J. Clin. Invest. 68: 932
46. Hazlot, A. et al (1988) J. Immunol. 141:547
47. Maliszewski, C.R. et al. (1985) J. Immunol 135:1929
48. Rigby, W.F.C. et al. (1984) Blood 64:1110-1115
49. Rigby, W.F.C. et al. (1985) Molec. Immun. 22:567-572
50. Simmons, D.L. et al. (1989) Blood 73:284-289
51. Martin, R. et al. (1992) Ann. Rev. Immunol. 10:153-187

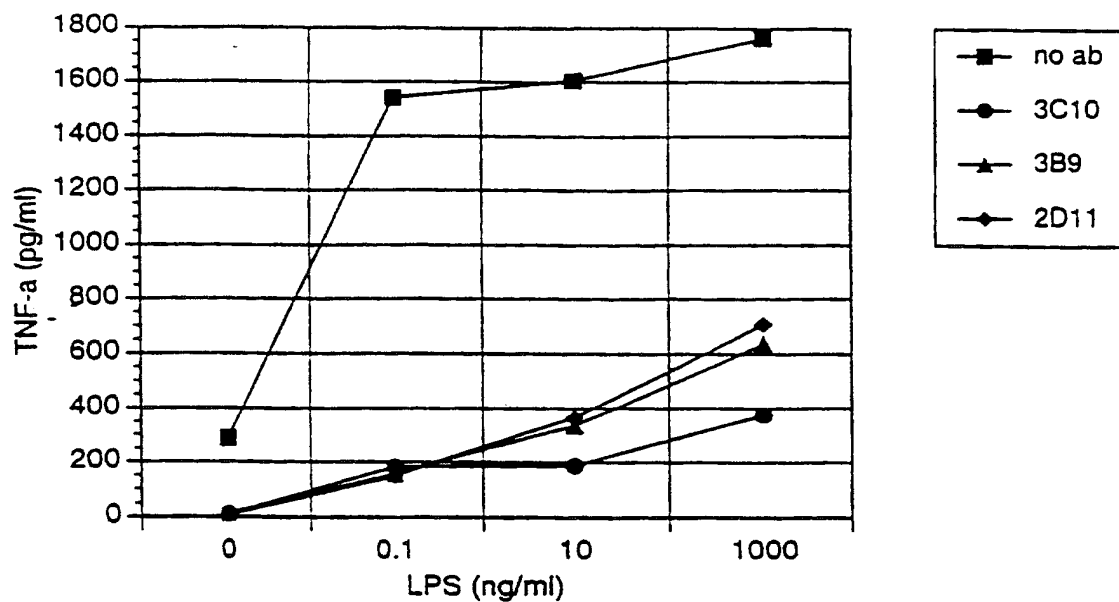
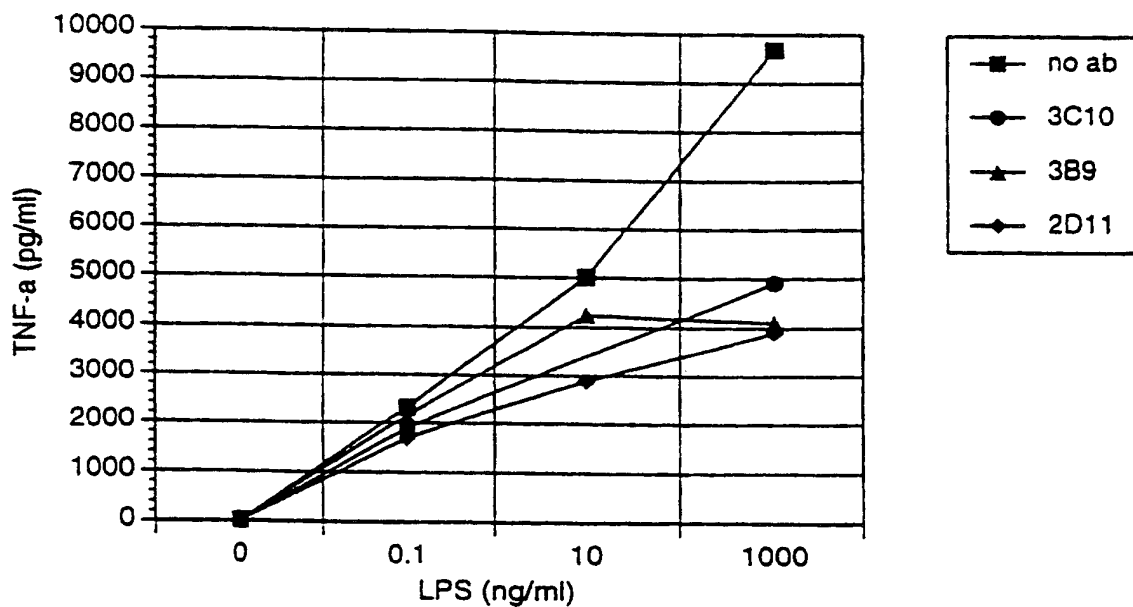
52. Gerlach, H. et al. (1993) Eur. J. Anaesthesiol. 10:273-285
53. Lauener, R. et al. 1990. Eur. J. Immunol. 20:2375-2381
54. Linsley et al. 1992. Science 792-795; Lenschow, D.J. et al., 1992. Science 257:789-792
55. Köntgen F. et al. 1993. International Immunology 5:957-964
56. Scheurich, P., et al. 1987, J. Immunol. 138:1786-1790
57. Yokota. S., et al. 1988, J. Immunol. 140:531-536

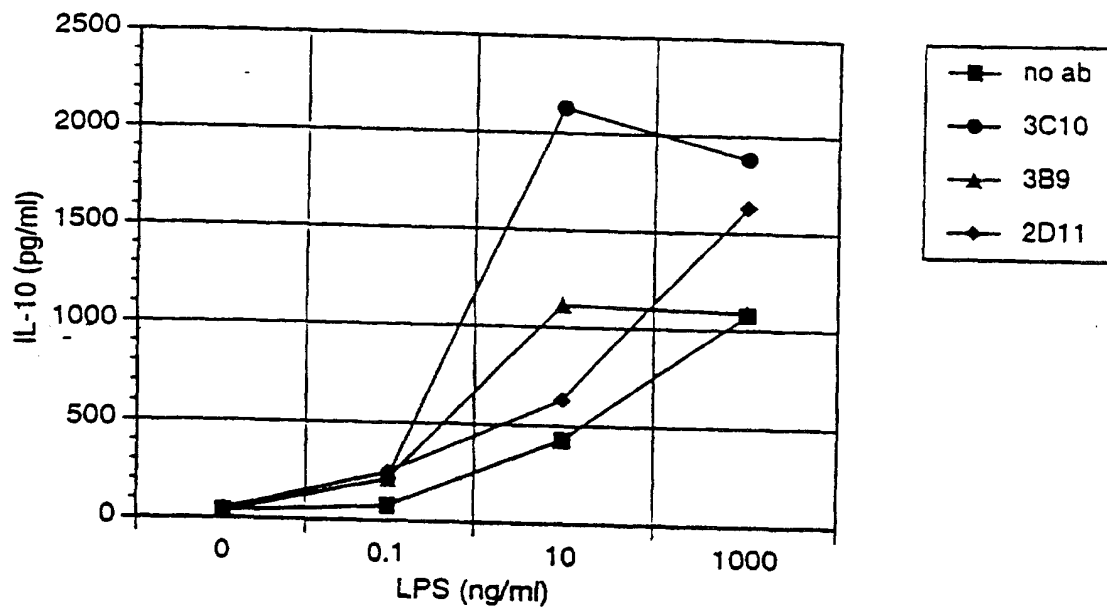
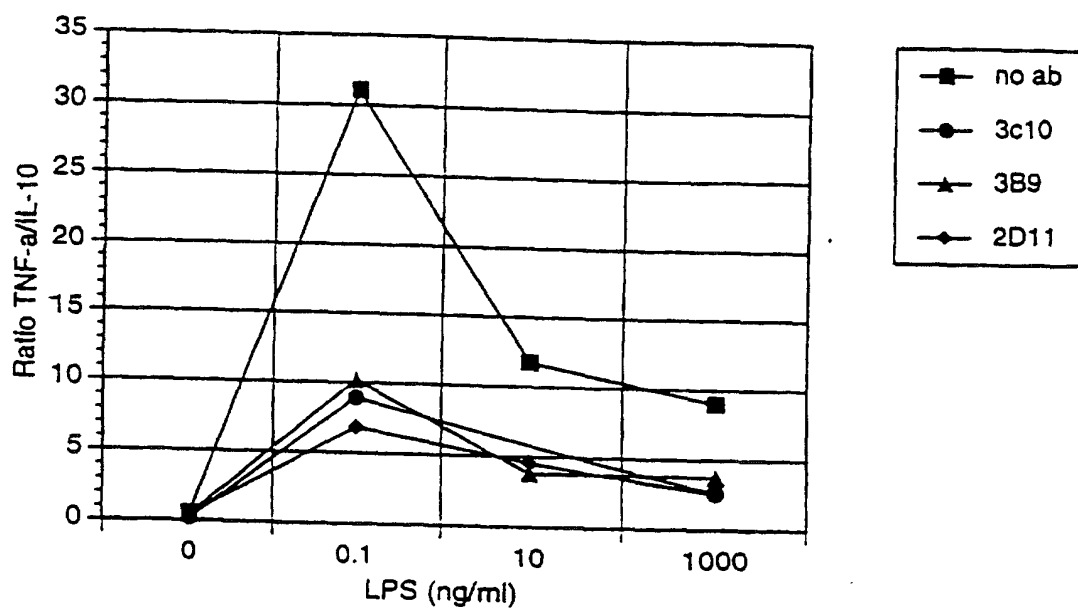
CLAIMS

1. Anti-CD14 antibodies for use in the induction and/or increase of interleukin-10 secretion.
- 5 2. Anti-CD14 antibodies for use in the prevention and/or treatment of inflammation by the induction and/or increase of interleukin-10 secretion.
3. Anti-CD14 antibodies as claimed in claim 2, wherein inflammation is a consequence of trauma, infection,
10 surgery, other medical and/or paramedical interventions, autoimmune reactions and diseases, degenerative processes, allergic disease, neoplastic processes, graft rejection, graft-versus-host-disease.
4. Anti-CD14 antibodies for use in
15 immunosuppression by the induction and/or increase of interleukin-10 secretion and/or induction of T cell tolerance and/or anergy.
5. Pharmaceutical composition for inducing and/or increasing interleukin-10 secretion, comprising anti-CD14
20 antibodies and/or fragments and/or modified versions thereof, together with a suitable excipient.
6. Pharmaceutical composition as claimed in claim 5 for use in the prevention and/or treatment of inflammation.
- 25 7. Pharmaceutical composition as claimed in claim 5 for use in immunosuppression.
8. Use of anti-CD14 antibodies for the preparation of a medicament for inducing and/or increasing interleukin-
10 secretion.
- 30 9. Use of anti-CD14 antibodies for the preparation of a medicament for downregulating TNF- α secretion.
10. Use of anti-CD14 antibodies for the preparation of a medicament for inducing T cell tolerance and/or anergy.
- 35 11. Use of anti-CD14 antibodies for the preparation of a medicament for the prevention and/or treatment of inflammation.

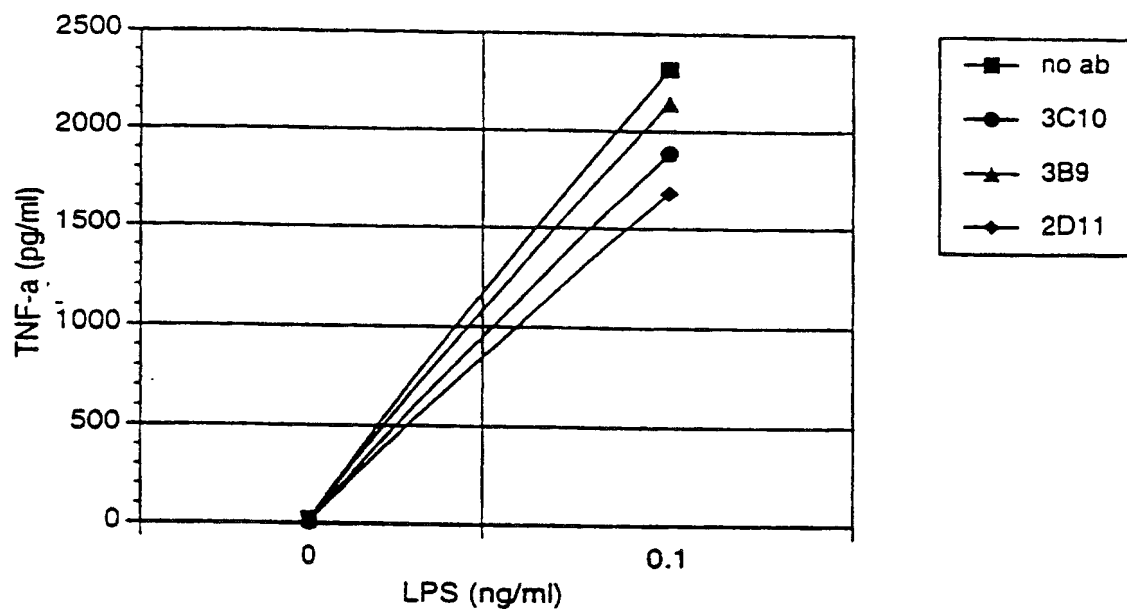
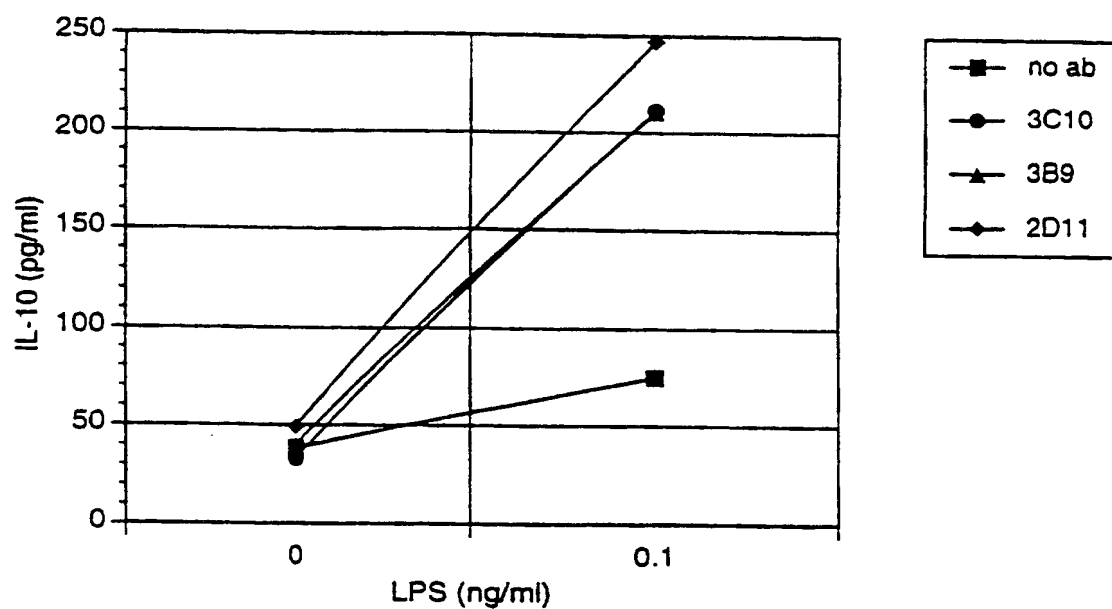
12. Use of anti-CD14 antibodies for the preparation of a medicament for immunosuppression.

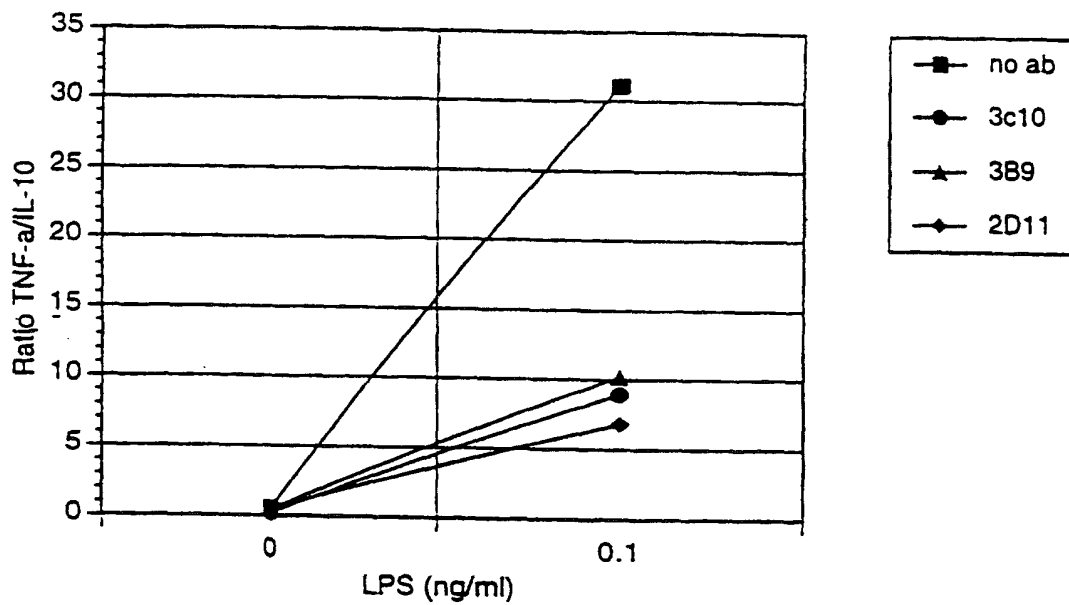
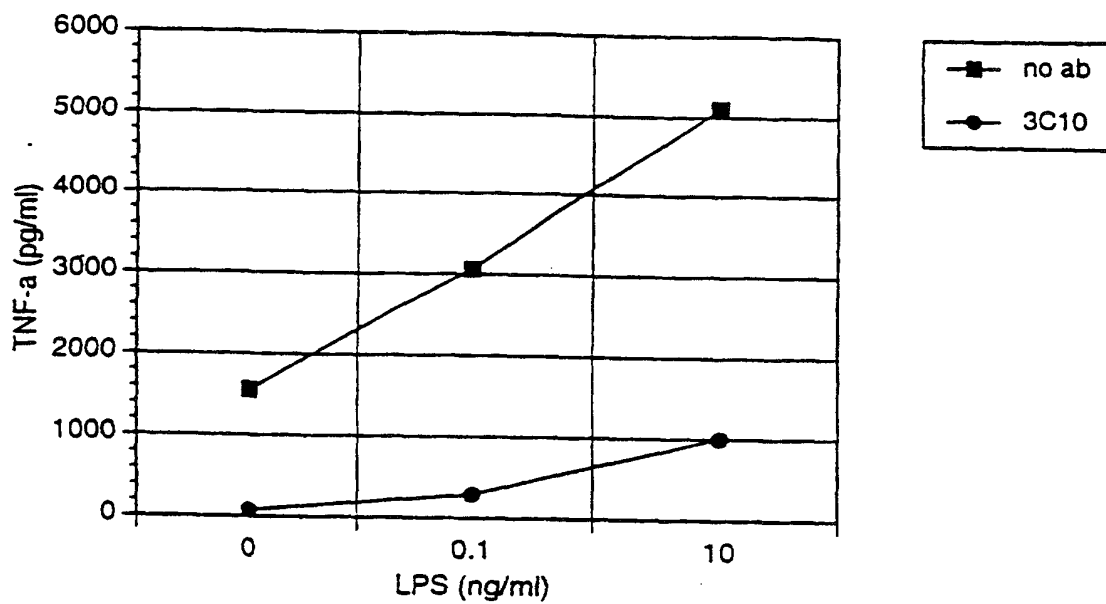
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FIG. 1FIG. 2a

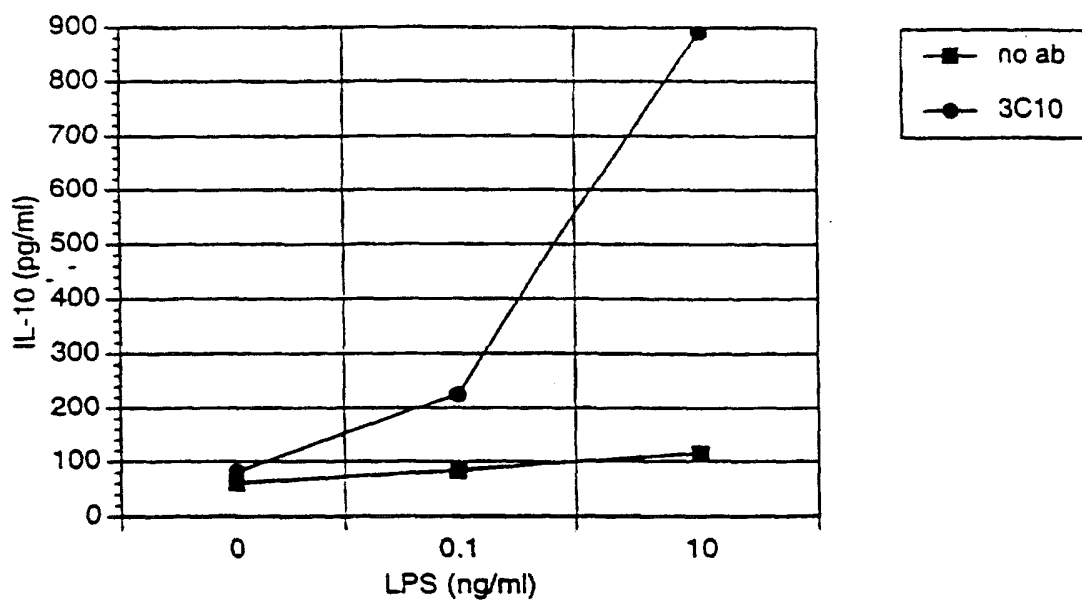
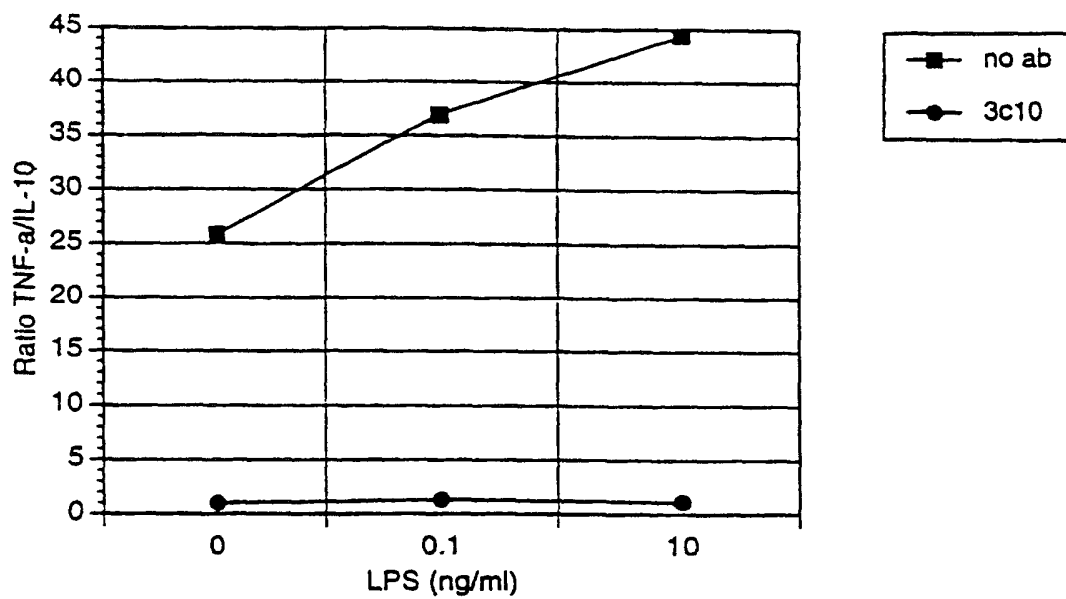
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FIG.2bFIG.2c

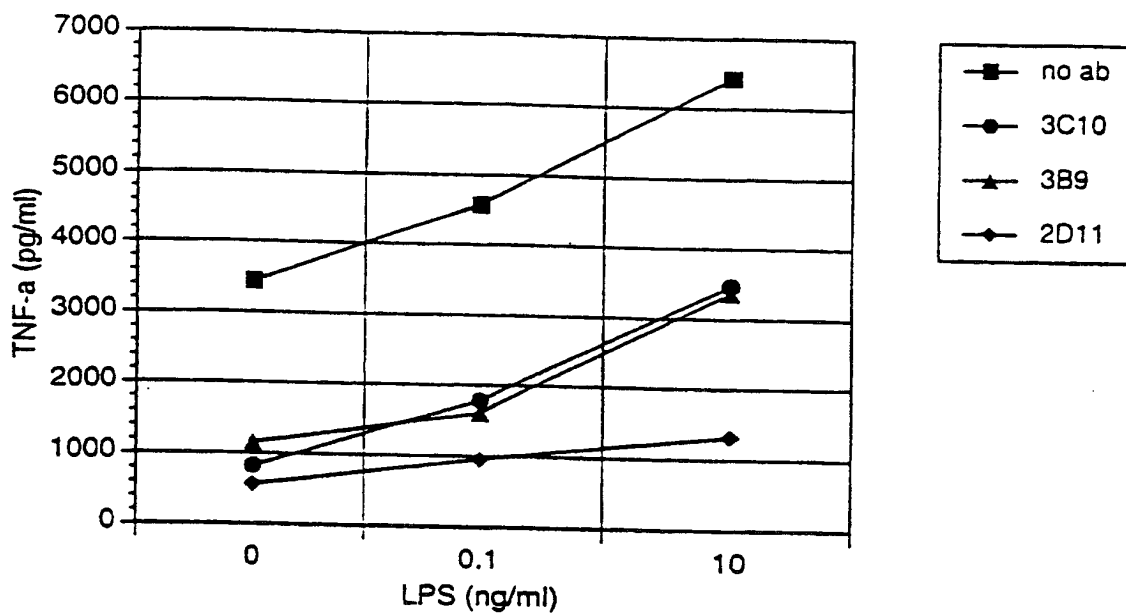
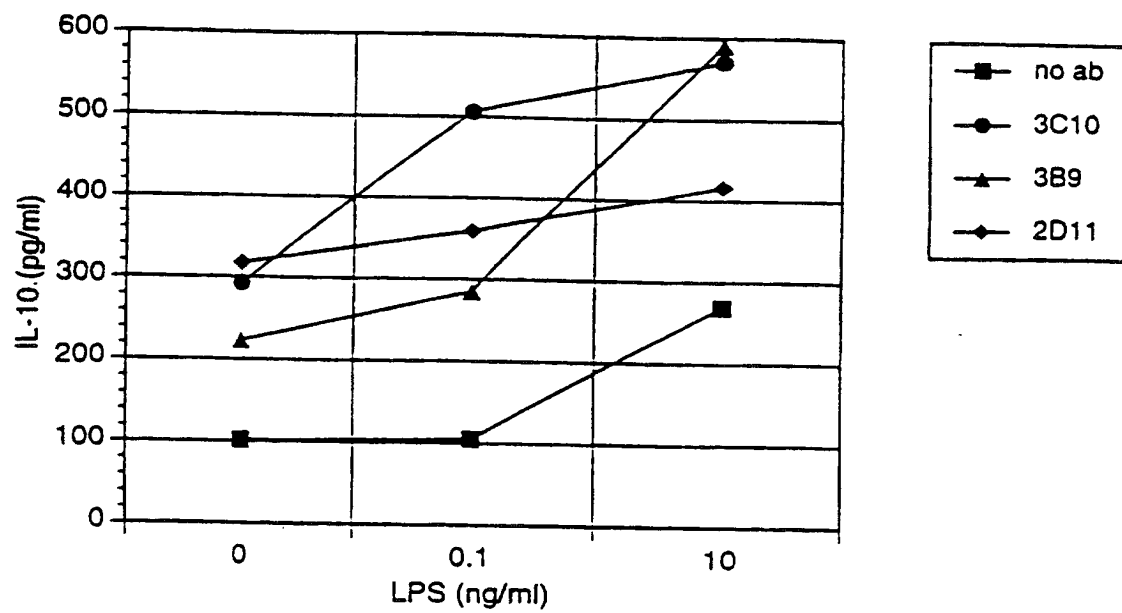
3/10

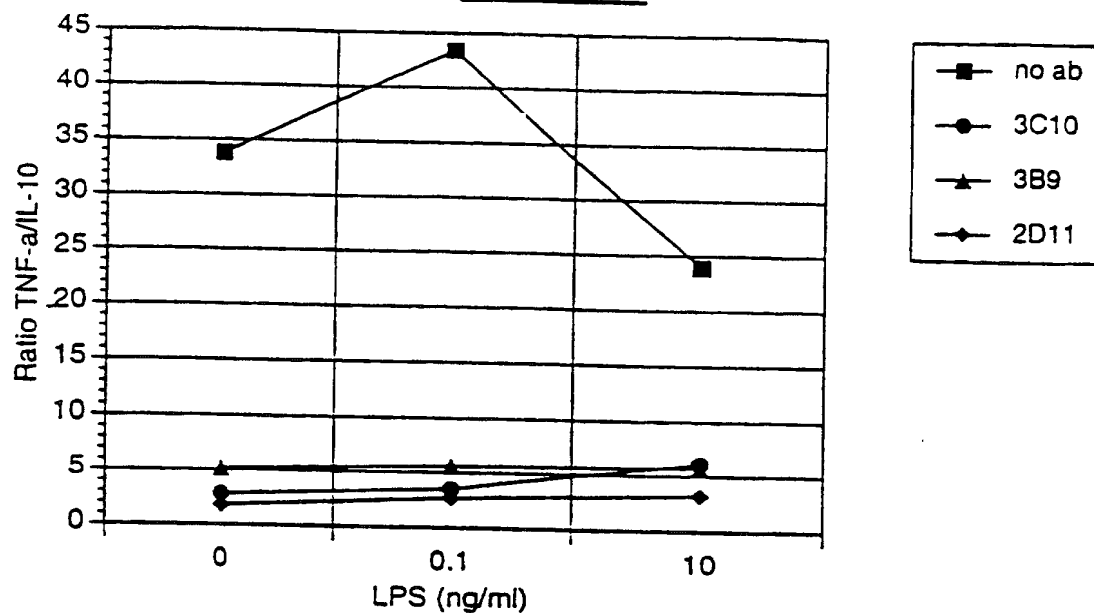
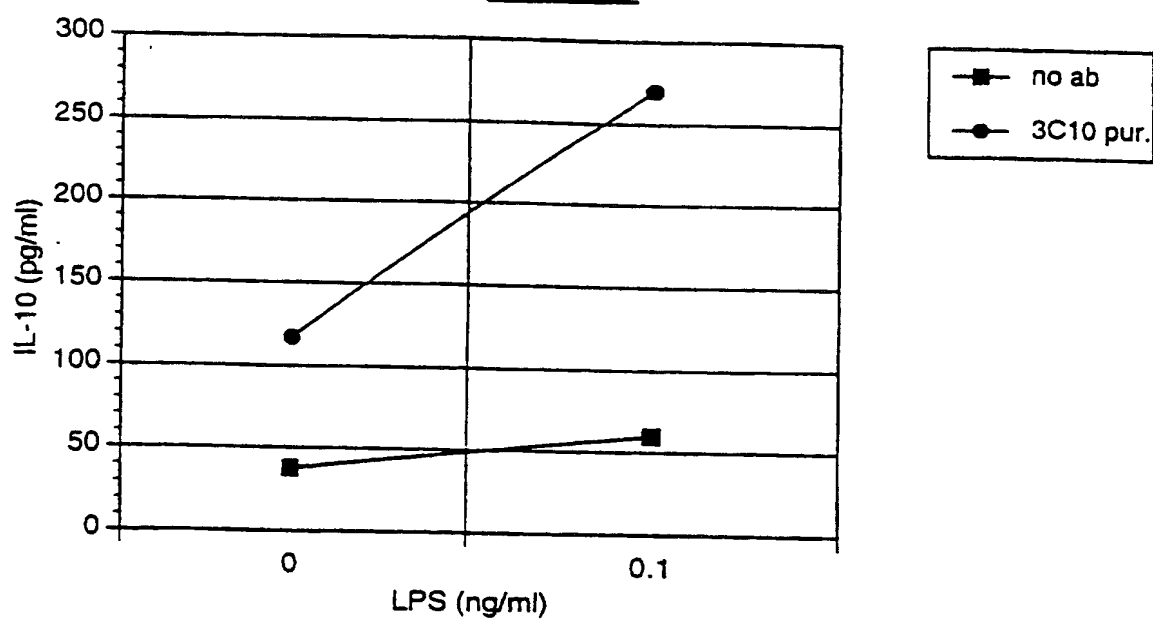
FIG.2a*FIG.2b*

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FIG. 2c*FIG. 3a

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FIG.3bFIG.3c

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FIG. 4aFIG. 4b

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FIG. 4cFIG. 5

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FIG. 6

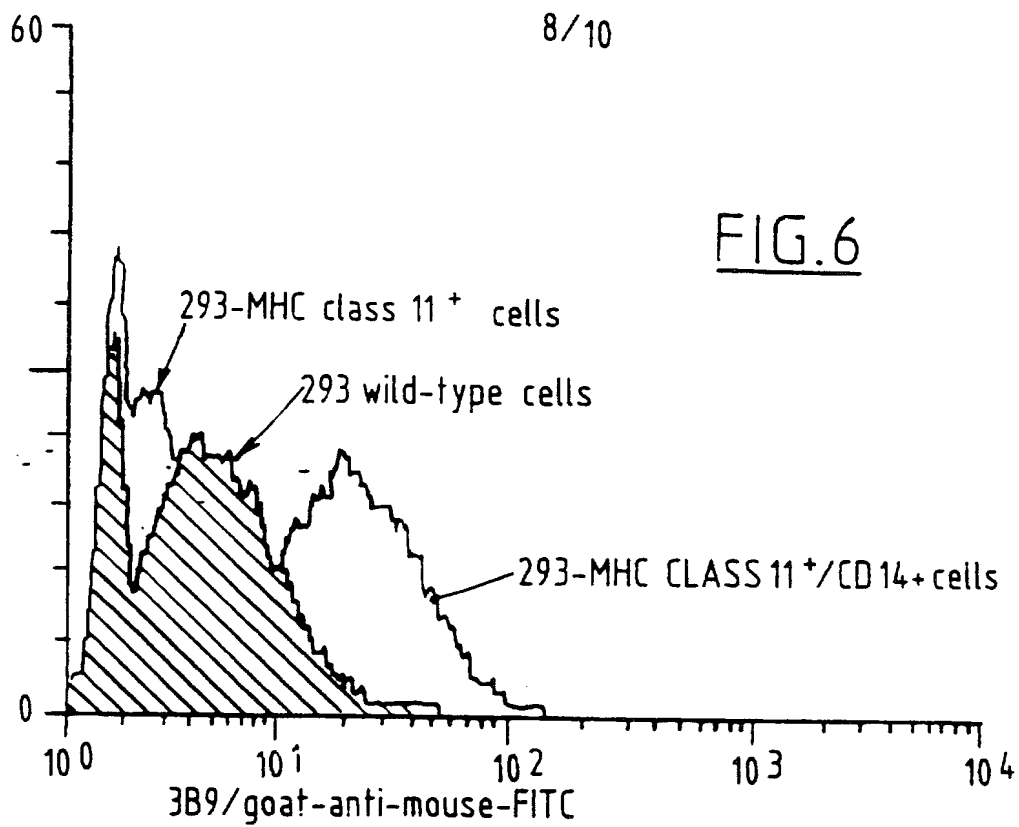
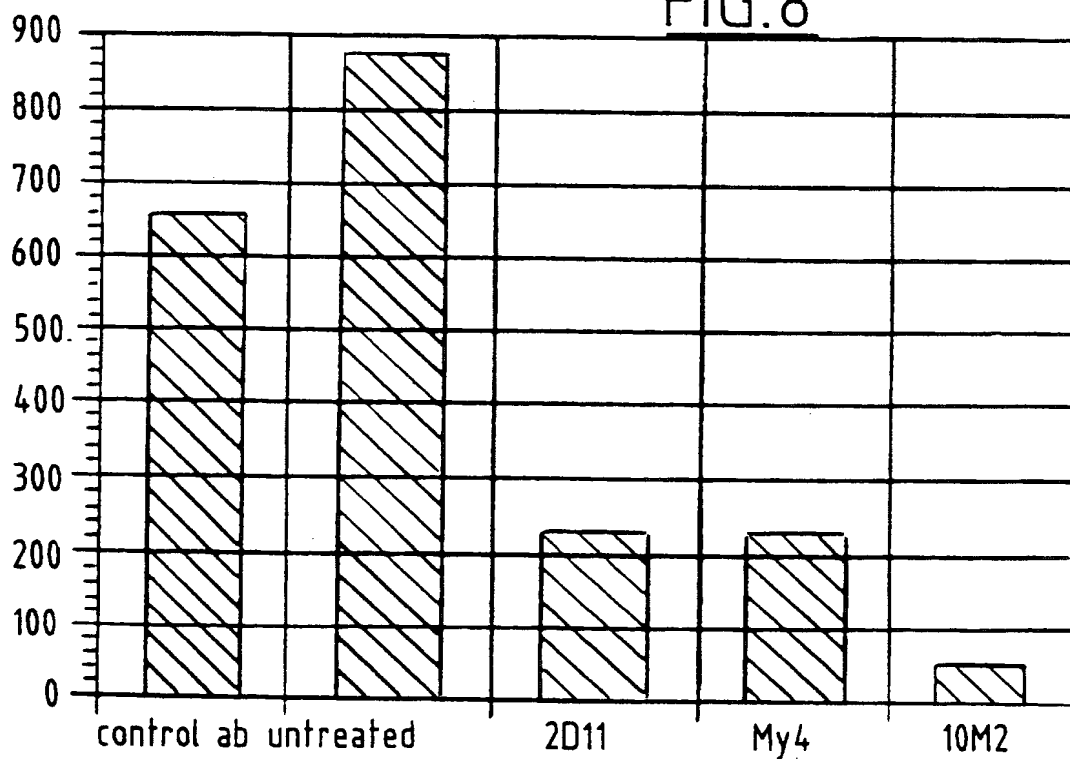
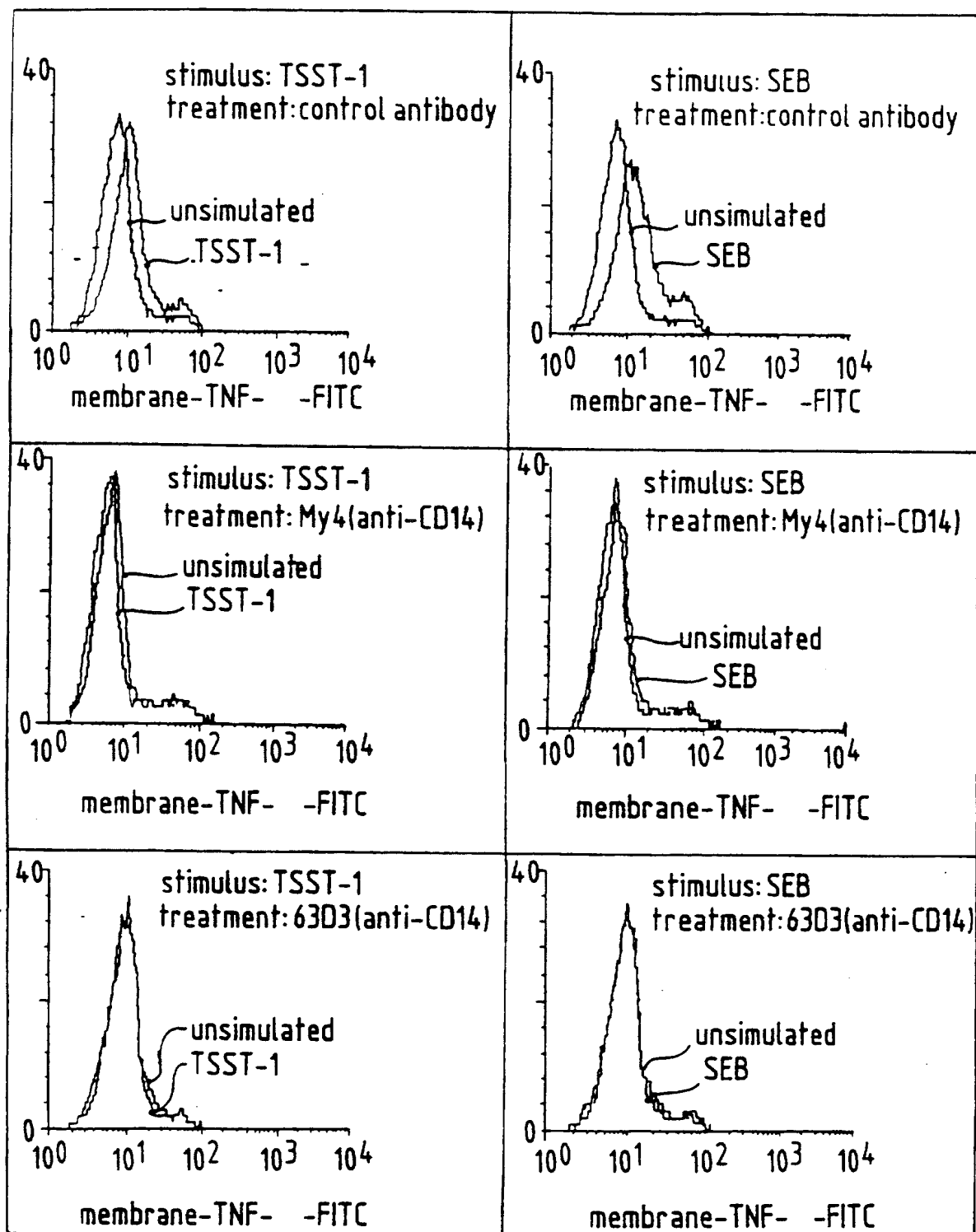


FIG. 8



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FIG. 7

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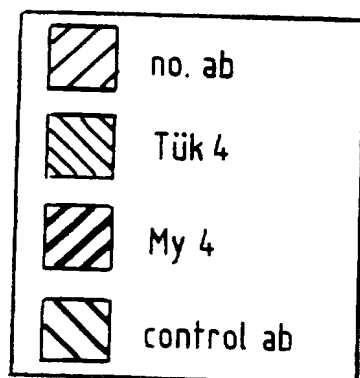
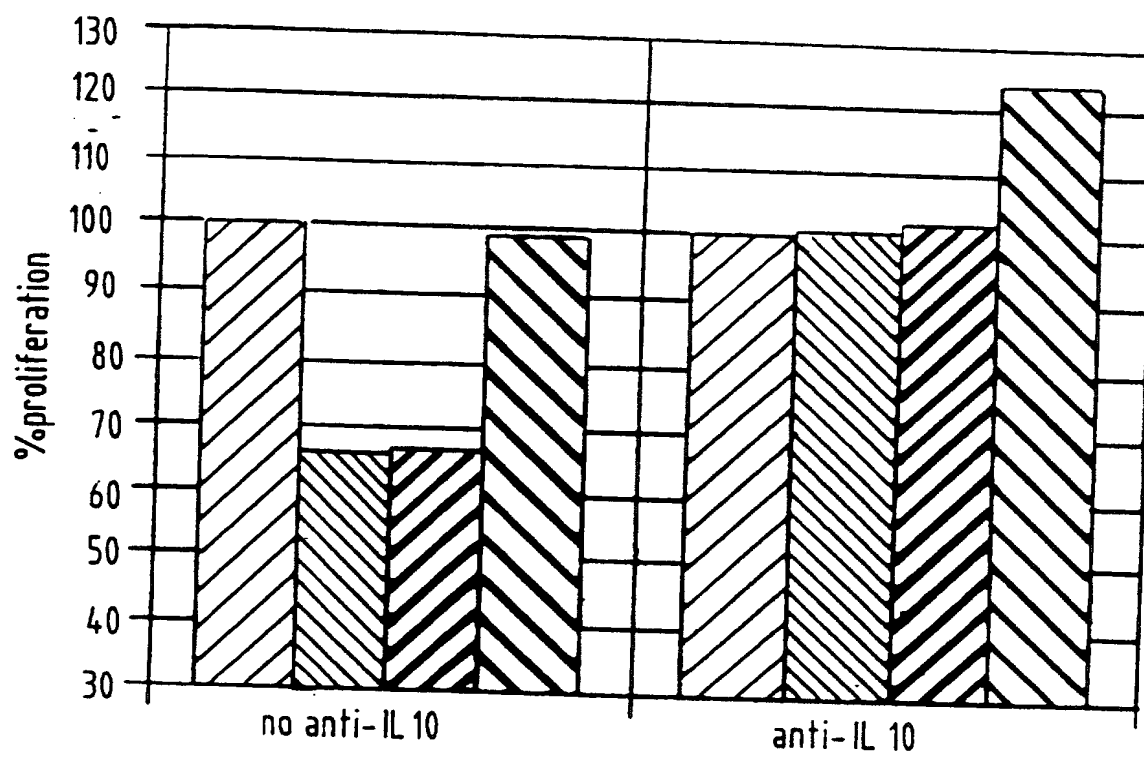


FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No

P-1/EP 96/01588

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/28 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THE JOURNAL OF IMMUNOLOGY, vol. 151, no. 7, 1 October 1993, BALTIMORE, MD, USA, pages 3829-3838, XP002009371 S. WEINSTEIN ET AL.: "Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14." see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	9,11,12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

25 July 1996

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PC/EP 96/01588

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SCIENCE, vol. 249, no. 4975, 21 September 1990, WASHINGTON, DC, USA, pages 1431-1433, XP002009372 S. WRIGHT ET AL.: "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein." see abstract see page 1432, left-hand column, line 36 - line 43 see figure 4</p>	9,11
X	<p>--- JOURNAL OF LEUKOCYTE BIOLOGY, vol. 57, no. 3, NEW YORK, NY, USA, pages 440-449, XP002009373 R. LANDMANN ET AL.: "LPS directly induces oxygen radical production in human monocytes via LPS binding protein and CD14." see abstract see page 445, left-hand column see page 447, left-hand column, line 47 - right-hand column, line 3</p>	9,11
X	<p>--- INFECTION AND IMMUNITY, vol. 63, no. 1, WASHINGTON, DC, USA, pages 253-258, XP002009374 T. PARKER ET AL.: "Reconstituted high-density lipoprotein neutralizes Gram-negative bacterial lipopolysaccharides in human whole blood." see abstract see figure 5</p>	9,11
X	<p>--- WO,A,91 01639 (SCRIPPS CLINIC AND RESEARCH FOUNDATION & ROCKEFELLER UNIVERSITY) 21 February 1991 see page 14, line 13 - page 15, line 4 see example 16 see claims</p>	9,11
X	<p>--- WO,A,94 28025 (THE SCRIPPS RESEARCH INSTITUTE) 8 December 1994 see page 21, line 23 - page 23, line 21 see example 4 see claims</p>	9,11
X	<p>--- THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, no. 6, 1 December 1993, NEW YORK, NY, USA, pages 2193-2200, XP000576151 J. PUGIN ET AL.: "A critical role for monocytes and CD14 in endotoxin-induced endothelial cell activation." see abstract</p> <p>-----</p>	9,11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/01588

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9101639	21-02-91	AU-B- 645515	20-01-94
		AU-B- 6146190	11-03-91
		CA-A- 2022429	02-02-91
		EP-A- 0485430	20-05-92
		GR-A- 90100582	30-12-91
		JP-T- 5501399	18-03-93

WO-A-9428025	08-12-94	AU-B- 7138494	20-12-94
		CA-A- 2163976	08-12-94
